410 Rec'd PCT/PTO 05 JUN 2000

PATENT

ATTORNEY DOCKET NO. LEBV.006.01US

09/555981

UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

In re application of: Noteborn and Danen-Van Oorschot	Examiner: Not yet assigned
International Application No.: PCT/NL98/00687	Art Unit: Not yet assigned
International Filing Date: December 3, 1998	**TRANSMITTAL FOR NEW PATENT APPLICATION UNDER 35 U.S.C. §371**
Priority Claimed: December 3, 1997	
For: **MOLECULES INTERACTING WITH APOPTIN**	

BOX PCT
Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.

CERTIFICATE OF EXPRESS MAILING

"Express Mail" Label No.: EL637192870US

Date of Deposit: 5 June, 2000

I hereby certify under 37 C.F.R. 1.10 that this correspondence is being deposited with the United States Postal Service as "Express Mail Post Office to Addressee" with sufficient postage on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, D.C., 20231.

Jennifer Wallsten

(Signature) Jennifer Wallsten

(Printed Name)

3. [X] This express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))

   a) [ ] is transmitted herewith (required only if not transmitted by the International Bureau).

   b) [X] has been transmitted by the International Bureau.

   c) [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).

6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))

   a) [ ] are transmitted herewith (required only if not transmitted by the International Bureau).

   b) [ ] have been transmitted by the International Bureau.

   c) [ ] have not been made; however, the time limit for making such amendments has NOT expired.

   d) [X] have not been made and will not be made.

8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. [X] An oath or declaration of the inventor(s) 35 U.S.C. 371(c)(4)) (unexecuted).

10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CGT 1.97 and 1.98.

12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. [X] A FIRST preliminary amendment.

[ ] A SECOND or SUBSEQUENT preliminary amendment.

14. [ ] A substitute specification.

15. [ ] A change of power of attorney and/or address letter.

16. [X] Other items or information.

[X] A copy of the application as published is enclosed.

[ ] This application is a CIP of

17. [X] The following fees are submitted:

Basic National Fee (37 CFR 1.492(a)(1)-(5)):

[ ]	USPTO was IPEA	
	[ ] All claims presented satisfied provisions of PCT Article 33(2) to (4)	$ 49.00/98.00
	[ ] All claims presented did not satisfy provisions PCT Article 33(2) to (4)	$360.00/720.00
[ ]	USPTO was ISA but not IPEA	$395.00/790.00
[X]	USPTO was neither ISA nor IPEA	
	[ ] Search report has not been prepared by the European Patent Office or the Japanese Patent Office	$535.00/1070.00
	[X] Search report has been prepared by the European Patent Office or the Japanese Patent Office	$465.00/930.00
	Basic Fee Amount	**= $ 930.00**

[X] Surcharge of $130.00 for furnishing the oath or declaration later than

[ ] 20 months

[X] 30 months

from the earliest claimed priority date (37 CFR 1.492(3)).

FOR:	Claims Filed	Extra Claims	Small Entity Rate	Fee	Other than a Small Entity Rate	Fee	Total Claim Fee
Total Claims	27	7	9.00		18.00	$	**$126.00**
Independent Claims	8	5	39.00		78.00	$	**$390.00**
Multiple Dependent Claims Presented			130.00		260.00	$	**$260.00**
TOTAL							**$776.00**

Total Claim Fee = $776.00

[ ] Verified Small Entity Statement enclosed with filing.

[ ] Processing fee of $130.00 for furnishing the English translation later than

[ ] 20 months

[ ] 30 months

from the earliest claimed priority date (37 CFR 1.492(f)).

[ ] Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property.

Total Fees = $1836.00

[ ] A check in the amount of $_____ to cover the above fees is enclosed.

[X] Please charge my Deposit Account No. 18-0020 in the amount of **$1,836.00** to cover the above fees.

A **duplicate** copy of this sheet is enclosed.

[X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 18-0020.

Respectfully submitted,

Date: 5 June, 2000

By: Jennifer Wahlsten
Jennifer L. Wahlsten, Ph.D.
Reg. No. 46,226

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09/555981
Rec'd PCT/PTO 05 JUN 2000

PATENT

ATTORNEY DOCKET NO. LEBV.006.01US

IN THE PATENT COOPERATION TREATY UNITED STATES RECEIVING OFFICE

In re Application of: Noteborn and Danen-Van Oorschot) Examiner: Not Yet Assigned
)
Serial No.: Not Yet Assigned) Art Unit: Not Yet Assigned
)
Filed: June 5, 2000) **PRELIMINARY AMENDMENT**
)
For: **MOLECULES INTERACTING WITH APOPTIN**)
)

BOX PCT
ATTN: US/RO
Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Applicant is submitting herewith a Preliminary Amendment in the above-referenced patent application. Prior to examination of the application, the Examiner is respectfully requested to enter the following amendments.

AMENDMENTS

In the Specification

On page 1, on the line following the title, insert --This application is a national stage filing under 35 U.S.C. §371 of PCT application PCT/NL98/00687 filed 3 December, 1998.--

CERTIFICATE OF EXPRESS MAILING

"Express Mail" Label No.: EL637192870US

Date of Deposit: 5 June, 2000

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Jennifer Wahlsten
(Signature)

Jennifer Wahlsten
(Printed Name)

In the Claims:

1. (Amended) A method of inducing apoptosis in a population of cells related to a pathological condition, said method comprising:

providing said population of cells with a [recombinant and/or isolated] nucleic acid molecule which is at least one of recombinant and isolated, wherein said nucleic acid molecule comprises a sequence encoding at least a functional part of a member [of] from the family of [Nmi-like] proteins selected from the group consisting of Nmi-like, [or at least a functional part of a member of the family of] HOU-like [proteins or at least a functional part of a member of the family of] and IFP35-like [proteins for use in the induction of apoptosis in a population of cells related to a pathological condition].

2. (Amended) [A use] The method according to claim 1 wherein said nucleic acid molecule comprises one of i) at least a [functional and specific] part of [the] a nucleic acid sequence [of] as shown in [figure] Figures 1, 2, 4, [or] 5 or 6; [encoding an amino sequence of figure 6] or ii) a nucleic acid sequence that is at least 60[, preferably 70, preferably 90] % homologous with at least one of said [functional and specific] nucleic acid [sequence] sequences shown in Figures 1, 2, 4, 5 or 6; or [comprising a sequence hybridizing] iii) a nucleic acid sequence that hybridizes to [any of the aforegoing] at least one of said nucleic acid sequences shown in Figures 1, 2, 4, 5 or 6 under stringent conditions; or iv) a nucleic acid sequence that encodes at least a functional part of an amino acid sequence as shown in Figures 3 or 7.

3. (Amended) [Use] The method according to claim 1 or 2, wherein said nucleic acid molecule comprises an expression vector.

4. (Amended) [Use] The method according to [anyone of the aforegoing claims] claim 1 or 2, [whereby] wherein said population of cells [are] is further provided with apoptosis inducing activity.

5. (Amended) [Use] The method according to claim 4, [whereby] wherein said apoptosis inducing activity is apoptin-like activity.

6. (Amended) [Use] The method according to [any of claims 1-5] claim 1 or 2, wherein said nucleic acid molecule is part of a gene delivery vehicle.

7. (Amended) A [recombinant and/or isolated] nucleic acid molecule which is at least one of recombinant and isolated, and [encoding] which encodes for [an] at least a functional part of a Nmi/Hou-like protein, said nucleic acid molecule comprising at least a [functional and/or specific] part of [the] a nucleic acid sequence [of] as shown in figure 1 or figure 2, or a nucleic acid sequence at least 60[, preferably 70, more preferably 80]% homologous therewith.

8. (Amended) A [recombinant and/or isolated] nucleic acid molecule which is at least one of recombinant and isolated, and [encoding] which encodes at least a functional part of an IFP35-like protein, said nucleic acid molecule comprising at least a [functional and/or specific] part of [the] a nucleic acid sequence [of] as shown in figure 4, [or] figure 5 or figure 6, [encoding at least a functional an/or specific part of the amino acid sequence of figure 6] or a nucleic acid sequence at least 60[, preferably 70, more preferably 80]% homologous therewith.

9. (Amended) An expression vector comprising a recombinant nucleic acid molecule according to claim 7 [and/]or 8.

10. (Amended) An expression vector according to claim 9 further comprising a nucleic acid sequence encoding for a protein with apoptotic activity.

11. (Amended) An expression vector according to claim 10 wherein said nucleic acid sequence encoding for a protein with apoptotic activity encodes apoptin or a functional fragment [and/]or equivalent thereof.

12. (Amended) A gene delivery vehicle comprising a recombinant nucleic acid molecule according to claim 7 or 8 [or an expression vector according to anyone of claims 9-11].

13. (Amended) A method of inducing apoptosis in a population of cells related to a pathological condition, said method comprising:

providing said population of cells with a sufficient amount of a [recombinant or isolated] proteinaceous substance which is at least one of recombinant and isolated, wherein said proteinaceous substance [comprising] comprises at least a functional part of a member of the family of Nmi/Hou-like proteins or at least a functional part of a member of the family of Hou-like proteins [for use in the induction of apoptosis in a population of cells related to a pathological condition].

14. (Amended) An Nmi/Hou-like proteinaceous substance [having] comprising at least one of i) a functional [and/or specific] part of [the] an amino acid sequence [of] as shown in figure 3; or [being] ii) a polypeptide encoded by [a functional and/or specific at least part of [the] a nucleic acid sequence [of] shown in figure 1 or figure 2; or [being] iii) an amino acid sequence that is at least 60[, preferably 70, preferably 80]% homologous to at least a functional [and/or specific] part of [the] an amino acid sequence [of] shown in figure 3; or [being] iv) an amino acid sequence that is at least 60[, preferably 70, preferably 80]% homologous to a protein encoded by at least a [functional an/or specific] part of [the] a nucleic acid sequence [of] shown in figure 1 or figure 2.

Cancel Claim 15.

16. (Amended) An IFP35-like proteinaceous substance [having] comprising at least one of i) a functional [and/or specific] part of [the] an amino acid sequence [of] shown in figure [6 or] 7; or [being] an amino acid sequence encoded by [a functional and/or specific] at least a part of [the] a nucleic acid sequence [of] shown in figure 4, [or] figure 5 or figure 6; or [being] iii) an amino acid sequence that is at least 60[, preferably 70, preferably 80]% homologous to at least a functional [and/or specific] part of [the] an amino acid sequence [of] shown in figure [6 or] 7; or [being] iv) an amino acid sequence that is at least 60[, preferably 70, preferably 80]% homologous to a protein encoded by at least a [functional and/or specific] part of [the] a nucleic acid sequence [of] shown in figure 4, [or] figure 5 or figure 6.

17. (Amended) A method for inducing apoptosis in cells, said method comprising providing said cells with Nmi/Hou-like protein activity and/or [IFP-35-like] IFP35-like activity together with apoptin-like activity.

18. (Amended) [Use of apoptin to find] A method of identifying proteinaceous substances associated with apoptosis, said method comprising:

identifying cDNA sequences that encode proteins that bind to apoptin.

Please add the following new claim:

--19. (New) A gene delivery vehicle comprising an expression vector according to claim 10.--.

REMARKS

Amendments

Claim 15 is canceled, Claims 1-14 and 16-18 are amended and new Claim 19 is added. The amendments were made so that the claims would recite appropriate alternative language, to eliminate multiple dependencies and to clarify references to nucleic acid and

amino acid sequences. Claims 1-6, 13 and 18 were amended to recite appropriate method claim language. Support for new Claim 19 is found in Claim 12.

No new matter is introduced by these amendments and the Examiner is respectfully requested to enter them.

CONCLUSION

If in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned at (650) 328-4400.

Respectfully submitted,

Dated: 5 June 2000

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09550394 - 060400

PATENT ATTORNEY DOCKET NO. LEBV.006.01US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Mathieu H. M. Noteborn *et al.*	) Examiner: Not Yet Assigned
	)
	) Art Unit: Not Yet Assigned
Serial No.: Not Yet Assigned	)
	) **VERIFIED STATEMENT**
Filed: June 5, 2000	) **(DECLARATION) CLAIMING**
	) **SMALL ENTITY STATUS**
For: Molecules interacting with apoptin	) **(37 C.F.R. §§ 1.9(f) & 1.27(c) –**
	) **SMALL BUSINESS CONCERN**

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I hereby declare that I am:

[ ] the owner of the small business concern identified below:

[X] an official of the small business concern empowered to act on behalf of the concern identified below:

Name of Concern: Leadd bv
Address of Concern: _____

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-

CERTIFICATE OF FIRST-CLASS MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first-class mail in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231 on JULY 26, 2000
(Date)

Signature: Alden

Printed Name: JESSICA ALDEA

time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled:

Molecules interacting with apoptin

by inventor(s) Mathieu H. M. Noteborn, Astrid AAM Danen-Van Oorschot, , ,

[ ] the specification filed herewith

[X] the specification filed June 5, 2000 as attorney docket number LEBV.006.01US.

[ ] Patent No. _____, issued _____

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a non-profit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

Name _____

Address _____

[ ] Individual [ ] Small business concern [ ] Non-profit organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(d)).

I hereby declare that all statements are made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may

jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Person Signing Dr Dirkjan Mahman
Title of Person Other than Owner Chief Executive Officer
Address of Person Signing Leaden BV PO Box 9503
Signature NL 2300 RA Leiden
Date 03 July 2000 The Netherlands

BRV: mfc

TOTAL P.10

MOLECULES INTERACTING WITH APOPTIN

The present invention relates to the field of apoptosis, as well as to the field of cancer diagnosis and treatment, and treatment and diagnosis of (auto-)immune diseases and other diseases related to regulation of apoptosis. The present invention specifically relates to molecules found to be involved in apoptotic pathways and their uses in inducing apoptosis in aberrant cells. The presently invented molecules have been identified using apoptin. Apoptin is a protein originally found in chicken anemia virus (CAV; Noteborn et al., 1991) and was originally called VP3. The apoptotic activity of this protein was discovered by the group of the present inventors (Noteborn et al., 1994).

Apoptosis is an active and programmed physiological process for eliminating superfluous, altered or malignant cells (Earnshaw, 1995, Duke et al., 1996). Apoptosis is characterized by shrinkage of cells, segmentation of the nucleus, condensation and cleavage of DNA into domain-sized fragments, in most cells followed by internucleosomal degradation. The apoptotic cells fragment into membrane-enclosed apoptotic bodies. Finally, neighbouring cells and/or macrophages will rapidly phagocytose these dying cells (Wyllie et al., 1980, White, 1996). Cells grown under tissue-culture conditions and cells from tissue material can be analysed for being apoptotic with agents staining DNA, as e.g. DAPI, which stains normal DNA strongly and regularly, whereas apoptotic DNA is stained weakly and/or irregularly (Noteborn et al., 1994, Telford et al., 1992).

The apoptotic process can be initiated by a variety of regulatory stimuli (Wyllie, 1995, White 1996, Levine, 1997). Changes in the cell survival rate play an important role in human pathogenesis, e.g. in cancer development and auto-immune diseases, which is caused by enhanced proliferation but also by decreased cell death (Kerr et al., 1994,

Paulovich, 1997). A variety of chemotherapeutic compounds and radiation have been demonstrated to induce apoptosis in tumor cells, in many instances via wild-type p53 protein (Thompson, 1995, Bellamy et al., 1995, Steller, 1995, McDonell et al., 1995).

Many tumors, however, acquire a mutation in p53 during their development, often correlating with poor response to cancer therapy. Transforming genes of tumorigenic DNA viruses can inactivate p53 by directly binding to it (Teodoro, 1997). An example of such an agent is the large T antigen of the tumor DNA virus SV40. For several (leukemic) tumors, a high expression level of the proto-oncogene Bcl-2 or Bcr-abl is associated with a strong resistance to various apoptosis-inducing chemotherapeutic agents (Hockenberry 1994, Sachs and Lotem, 1997).

For such cancers (representing more than half of the tumors) alternative anti-tumor therapies are under development based on induction of apoptosis independent of p53 (Thompson 1995, Paulovich et al., 1997). One has to search for the factors involved in induction of apoptosis, which do not need p53 and/or can not be blocked by Bcl-2/Bcr-abl-like anti-apoptotic activities. These factors might be part of a distinct apoptosis pathway or might be (far) downstream to the apoptosis inhibiting compounds.

Apoptin is a small protein derived from chicken anemia virus (CAV; Noteborn and De Boer, 1995, Noteborn et al., 1991, Noteborn et al., 1994), which can induce apoptosis in human malignant and transformed cell lines, but not in untransformed human cell cultures. In vitro, apoptin fails to induce programmed cell death in normal lymphoid, dermal, epidermal, endothelial and smooth-muscle cells. However, when normal cells are transformed they become susceptible to apoptosis by apoptin. (Danen-van Ooschot, 1997 and Noteborn, 1996). Long-term expression of apoptin in normal human fibroblasts revealed that apoptin has no toxic or transforming activity in these cells.

In normal cells, apoptin was found predominantly in the cytoplasm, whereas in transformed or malignant cells i.e. characterized by hyperplasia, metaplasia or dysplasia, it was located in the nucleus, suggesting that the localization of apoptin is related to its activity (Danen-van Oorschot et al. 1997).

Apoptin-induced apoptosis occurs in the absence of functional p53 (Zhuang et al., 1995a), and cannot be blocked by Bcl-2, Bcr-abl (Zhuang et al., 1995), the Bcl-2-associating protein BAG-1 and not by the caspase-inhibitor cowpox protein CrmA (Danen-Van Oorschot, 1997a, Noteborn, 1996).

Therefore, apoptin is useful for the destruction of tumor cells, or other hyperplasia, metaplasia or dysplasia which have become resistant to (chemo)therapeutic induction of apoptosis, due to the lack of functional p53 and (over)-expression of Bcl-2 and other apoptosis-inhibiting agents (Noteborn et al., 1997).

The fact that apoptin does not induce apoptosis in normal human cells, at least not in vitro, suggests that a toxic effect of apoptin treatment in vivo will be very low. Noteborn et al. (1997) have provided evidence that adenovirus expressed apoptin does not have an acute toxic effect in vivo. In addition, in nude mice it was shown that apoptin has a strong anti-tumor activity.

It appears, that even pre-malignant, minimally transformed cells, are sensitive to the death-inducing effect of apoptin. In addition, Noteborn and Zhang (1997) have shown that apoptin-induced apoptosis can be used as diagnosis of cancer-prone cells and treatment of cancer-prone cells.

Knowing that apoptin is quite safe in normal cells, but that as soon as a cell becomes transformed and/or immortalized (the terms may be used interchangeable herein) the present inventors designed some uses based on the identification of compounds involved in the apoptin-induced apoptotic cascade. These compounds are factors of an

apoptosis pathway, which is specific for transformed cells. Therefore, these proteins are very important compounds in new treatments and diagnosis for diseases related with aberrancies in the apoptotic process, such as cancer and auto-immune diseases.

Proteins found associating with apoptin include members of the family of Nmi/Hou-like and IFP-like proteins.

Thus the invention provides a recombinant and/or isolated nucleic acid molecule encoding at least a functional part of a member of the family of Nmi-like proteins or at least a functional part of a member of the family of Hou-like proteins or at least a functional part of a member of the family of IFP35-like proteins for use in the induction of apoptosis in a population of cells related to a pathological condition.

As explained herein the expression of Hou is connected to oncogenes and has been found to be high in certain transformed cells. These are typically the cells that can be induced to go into apoptosis by apoptotic agents such as apoptin. Typically providing a cell with Hou-like activity will therefor increase the chance of inducing apoptosis in such a cell. IFP35-like proteins are involved in transporting apoptotic substances to the nucleus of cells. Under influence of for instance interferons these proteins localize in the nucleus. Therefor IFP-like activity is used to get apoptin-like activity into the nucleus, which is important for the induction of apoptosis, for instance through Hou-like proteins. The Hou-like activity or Nmi-like activity is defined herein as any molecule capable of exerting the sanme or a similar function as the original Hou-like (Nmi-like) protein. The same definition goes for IFP-activity. Typically such a molecule can be encoded by a nucleic acid molecule which comprises at least a functional and specific part of the sequence of figure 1, 2, 4 or 5 or encoding an amino sequence of figure 6 or a sequence at least 60, preferably 70, preferably 90 % homologous with said functional and

specific sequence or comprising a sequence hybridizing to any of the aforegoing sequences under stringent conditions. In order to be able to express the Hou-like activity and/or the IFP-like activity it is preferred to have an expression vector encoding said activity. Expression vectors are nucleic acid molecules which can be brought into cells, or transfect cells themselves and which have the machinery (together with the machinery of the host cell)to express proteins encoded on the expression vector when present in a cell.

It is preferred that cells which are provided, according to the invention, with Hou-like activity and/or IFP-like activity, are also provided with apoptosis inducing activity, preferably apoptin-like activity, which is defined along the same lines as Hou-like activity. In order to get the activity into the cells in which apoptosis has to be induced it is possible and preferred to use a gene delivery vehicle. A gene delivery vehicle is a means to transport a nucleic acid molecule capable of expressing the wanted activity in a host cell into said host cell. Gene delivery vehicles are known in the art. They include for instance recombinant viruses such as adenoviruses and retroviruses, but also non-viral vehicles such as polymers and liposomes have been suggested. Methods of targeting gene delivery vehicles to target cells are also known in the art and need not be elaborated herein. The invention also provides the newly identified molecules themselves, both the nucleic acid molecules (meaning DNA coding and/or non coding strands as well as RNA) and the proteinaceous molecules (peptides, polypeptides, glycoproteins and associations between prtoeins and RNA's and the like). Based on the given sequences other familymembers of the Hou/Nmi and IFP families will be identified having the same or similar function. Typically such molecules will have high homology to the sequences given herein.

For nucleic acid molecules the homology is exppected to be at least 60, preferably 70, more preferably 80%. therewith.

These nucleic acid molecules can of course again be incorporated into expression vectors as mentioned hereinbefore. Preferably these expression vectors also encode apoptotic activity, preferably apoptin or a functional fragment and/or equivalent thereof.

These expression vectors can again be made into gene delivery vehicles.

The invention also provides the recombinant or isolated proteinaceous substance comprising at least a functional part of a member of the family of Nmi/Hou-like proteins or at least a functional part of a member of the family of Hou-like proteins for use in the induction of apoptosis in a population of cells related to a pathological condition and an Nmi/Hou-like proteinaceous substance having at least a functional and/or specific part of the sequence of figure 3 or being encoded by a functional and/or specific part of the sequence of figure 1 or figure 2 or being at least 60, preferably 70, preferably 80% homologous to at least a functional and/or specific part of the sequence of figure 3 or being at least 60, preferably 70, preferably 80% homologous to a protein encoded by at least a functional and/or specific part of the sequence of figure 1 or figure 2 and an IFP35-like proteinaceous substance having at least a functional and/or specific part of the sequence of figure 6 or 7 or being encoded by a functional and/or specific part of the sequence of figure 4 or figure 5 or being at least 60, preferably 70, preferably 80% homologous to at least a functional and/or specific part of the sequence of figure 6 or 7 or being at least 60, preferably 70, preferably 80% homologous to a protein encoded by at least a functional and/or specific part of the sequence of figure 4 or figure 5.

A functional part in this document means having the same or similar activity (although the amount of activity may differ). A specific part herein means a part of sufficient size to be specific for the protein or nucleic acid or to be of sufficient size to distinguish the protein from another

protein immunologically. The proteins disclosed herein can for instance also be used to identify further components of the apoptotic pathway.

The reason for bringing IFP-like activity and/or Hou-like activity together with apoptotic activity is of course to induce aberrant cells to go into apoptosis. Thus the invention also provides a method for inducing apoptosis in cells comprising providing said cells with Nmi/Hou-like protein activity and/or IFP-35-like activity together with apoptin-like activity.

The invention further provides a method for inducing apoptosis through interference with the function of Nmi/Hou-like proteins (interchangeably referred as Hou-, Nmi- or Nmi/Hou-like proteins).

The invention provides an anti-tumor therapy based on the interference with the function of Hou or Hou-like proteins. The fact that Hou or Hou-like proteins are abundantly present in tumor cells in combination with highly expressed oncogenes, - which are activated by Hou or Hou-like proteins -, make Hou and/or Hou-like proteins very important targets of an anti-tumor agent/therapy.

The invention provides the mediator of apoptin-induced apoptosis, which is tumor-specific.

The invention provides a therapy for cancer, auto-immune diseases or related diseases which is based on Hou-like proteins in combination with apoptin and/or apoptin-like compounds.

The invention further provides a method for inducing apoptosis through interference with the function of IFP35-like proteins.

The invention provides an anti-tumor therapy based on the interference with the function of IFP35 or IFP35-like proteins.

The invention provides IFP35 as a mediator of apoptin-induced apoptosis, which is tumor-specific.

The invention provides a therapy for cancer, auto-immune diseases or related diseases which is based on IFP35 or IFP35-like proteins in combination with apoptin and/or apoptin-like compounds.

The invention provides a therapy based on the combination of apoptin-associating proteins Hou and IFP35.

The invention further provides a method for inducing apoptosis through interference with the function of IFP35 and Hou or IFP35-and Hou-like proteins.

The invention provides an anti-tumor therapy based on the interference with the function of IFP35 or IFP35-like proteins, in combination with Hou or Hou-like proteins.

The invention provides IFP35 or IFP35-like in combination with Hou or Hou-like proteins as mediators of apoptin or apoptin-like induction of apoptosis, which is tumor-specific.

The invention provides a therapy for cancer, auto-immune diseases or related diseases which is based on the combination of IFP35 or IFP35-like and Hou or Hou-like proteins in combination with apoptin and/or apoptin-like compounds.

Furthermore, the invention provides a diagnosis, based on Hou or Hou-like proteins, which can determine whether cells are susceptible for apoptin-induced apoptosis. This means that patients with tumors having up-regulated Hou or Hou-like activity are optimal candidates for an anti-cancer therapy based on apoptin.

Furthermore, the invention provides a diagnosis, based on Hou or Hou-like proteins in combination with IFP35 or IFP35-like proteins, which can determine whether cells are susceptible for apoptin-induced apoptosis. This means that patients with tumors having up-regulated Hou or Hou-like activity in combination with IFP35 or IFP35-like activity are optimal candidates for an anti-cancer therapy based on apoptin.

The invention will be explained in more detail in the following experimental part. This only serves for the purpose of illustration and should not be interpreted as a limitation of the scope of the invention.

EXPERIMENTAL PART

The inventors have used the yeast-2 hybrid system (Durfee et al., 1993) to identify apoptin-associating cellular compounds, which are essential in the induction of apoptosis. The used system is an in vivo strategy to identify human proteins capable of physically associating with apoptin. It has been used to screen cDNA libraries for clones encoding proteins capable of binding to a protein of interest (Fields and Song, 1989, Yang et al., 1992).

Construction of pGBT9-VP3

For the construction of the bait plasmid, which enables the identification of apoptin-associating proteins by means of a yeast-two-hybrid system, plasmid pET-16b-VP3 (Noteborn, unpublished results) was treated with NdeI and BamHI. The 0.4 kb NdeI-BamHI DNA fragment was isolated from low-melting-point agarose.

Plasmid pGBT9 (Clontech Laboratories, Inc, Palo Alto, USA) was treated with the restriction enzymes EcoRI and BamHI. The about 5.4-kb DNA fragment was isolated and ligated to an EcoRI-NdeI linker and the 0.4-kb DNA fragment containing the apoptin-encoding sequences starting from its own ATG-initiation codon. The final construct containing a fusion gene of the GAL4-binding domain sequence and apoptin under the regulation of the yeast promoter ADH was called pGBT-VP3 and was proven to be correct by restriction-enzyme analysis and DNA-sequencing according to the Sanger method (1977).

All cloning steps were essentially carried out as described by Maniatis et al. (1992). The plasmid pGBT-VP3 was

prurified by centrifugation in a CsCl gradient and column chromatography in Sephacryl S500 (Pharmacia).

GAL4-activation domain-tagged cDNA library

The expression vector pACT, containing the cDNAs from Epstein-Barr-virus-transformed human B cells fused to the GAL4 transcriptional activation domain, was used for detecting apoptin-associating proteins. The pACT c-DNA lbrary is derived from the lambda-ACT cDNA library, as described by Durfee et al. 1993.

Bacterial and Yeast strains

The E.coli strain JM109 was the transformation recipient for the plasmid pGBT9 and pGBT-VP3. The bacterial strain electromax/DH10B was used for the transformation needed for the recovery the apoptin-associating pACT-cDNAs, and was obtained from GIBCO-BRL, USA.

The yeast strain Y190 was used for screening the cDNA library, and all other transformations which are part of the used yeast-two-hybrid system.

Media

For drug selections Luria Broth (LB) plates for E.coli were supplemented with ampicillin (50 microgram per ml). Yeast YPD and SC media were prepared as described by Rose et al. (1990).

Transformation of competent yeast strain Y190 with plasmids pGBT-VP3 and pACT-cDNA and screening for beta-galactosidase activity.

The yeast strain Y190 was made competent and transformed according to the methods described by Klebe et al. (Klebe et al., 1983). The yeast cells were first transformed with pGBT-VP3 and subsequently transformed with pACT-cDNA, and these transformed yeast cells were grown on histidine-minus plates, also lacking leucine and tryptophan.

Hybond-N filters were layed on yeast colonies , which were histidine-positive and allowed to wet completely. The filters were lifted and submerged in lquid nitrogen to permeabilize the yeast cells. The filters were thawed and layed with the colony side up on Whattman 3MM paper in a petridish with Z-buffer (Per liter: 16.1 gr $Na_2HPO_4.7H_2O$, 5.5 gr $NaH_2PO_4.H_2O$, 0.75 gr KCl and 0,246 gr $MgSO_4.7H_2O$, pH 7.0) containing 0.27% beta-mercapto-ethanol and 1 mg/ml X-gal. The filters were incubated for at least 15 minutes or during night.

Recovery of plasmids from yeast

Total DNA from yeast cells, which were histidine- and beta-galactosidase-positive, was prepared by using the glusulase-alkaline lysis method as described by Hoffman and Winston (1987) and used to transform Electromax/DH10B bacteria via electroporation using a Bio-Rad GenePulser according the manufacturer's specifications.

Transformants were plated on LB media containing ampicillin.

Isolation of apoptin-associating pACT clones

By means of colony-filter assay the colonies were lysed and hybridized to a radioactive-labeled 17-mer oligomer, which is specific for pACT (see also section Sequence analysis).

Plasmid DNA was isolated from the pACT-clones, and by means of XhoI digestion analysed for the presence of a cDNA insert.

Sequence analysis

The subclones containing the sequences encoding apoptin-associating proteins were sequenced using dideoxy NTPs according to the Sanger method which was performed by Eurogentec, Nederland BV (Maastricht, The Netherlands). The

used sequencing primer was a pACT-specific 17-mer comprising of the DNA-sequence 5'-TACCACTACAATGGATG-3'.

The sequences of the apoptin-associating proteins were compared with known gene sequences from the EMBL/Genbank.

Results and discussion

Apoptin induces specifically apoptosis in transformed cells, such as cell lines derived from human tumors. To identify the essential compounds in this cell-transformation-specific and/or tumor-specific apoptosis pathway, a yeast genetic screen was carried out.

We have used a human cDNA library, which is based on the plasmid vector pACT containing the complete cDNA copies made from Epstein-Barr virus-transformed human B cells (Durfee et al., 1993).

Construction of a bait plasmid expressing a fusion gene product of GAL4-DNA-binding domain and apoptin

To examine the existence of apoptin-associating proteins in the human transformed/tumorigenic cDNA library, a so-called bait plasmid had to be constructed.

To that end, the complete apoptin-encoding region, flanked by about 40 basepairs downstream from the apoptin gene, was cloned in the multiple cloning site of plasmid pGBT9.

The final construct, called pGBT-VP3, was analysed by restriction-enzyme analysis and sequencing of the fusion area between apoptin and the GAL4-DNA-binding domain.

A gene(fragment) encoding an apoptin-associating protein is determined by transactivation of a GAL4-responsive promoter in yeast.

The apoptin gene is fused to the GAL4-DNA-binding domain of plasmid pGBT-VP3, whereas all cDNAs derived from the transformed human B cells are fused to the GAL4-activation domain of plasmid pACT. If one of the cDNAs will bind to

apoptin, the GAL4-DNA-binding domain will be in the vicinity of the GAL4-activation domain resulting in the activation of the GAL4-responsive promoter, which regulates the reporter genes HIS3 and LacZ.

The yeast clones containing plasmid expressing apoptin and a plasmid expressing an apoptin-associating protein fragment can grow on a histidine-minus medium and will stain blue in a beta-galactosidase assay. Subsequently, the plasmid with the cDNA insert encoding the apoptin-associating protein can be isolated and characterized.

Before we could do so, however, we have determined that transformation of yeast cells with pGBT-VP3 plasmid alone or in combination with an empty pACT vector, did not result in the activation of the GAL4-responsive promoter.

Identification of apoptin-associating proteins encoded by cDNAs derived from a human transformed B cell line.

We have found yeast colonies, which upon transformation with pGBT-VP3 and pACT-cDNA were able to grow on a histidine-minus medium (also lacking leucine and tryptophan) and stained blue in a beta-galactosidase assay. These results indicate that the observed yeast colonies contain besides the bait plasmid pGBT-VP3 also a pACT plasmid encoding a potential apoptin-associating protein.

Plasmid DNA was isolated from these positive yeast colonies, which were transformed in bacteria. By means of a filter-hybridization assay using a pACT-specific labeled DNA-probe, the clones containing pACT plasmid could be determined. Subsequently, pACT DNA was isolated and digested with restriction enzyme XhoI, which is indicative for the presence of a cDNA insert. Finally, the pACT plasmids with a cDNA insert were sequenced.

Description of apoptin-associating proteins

The yeast genetic screen for apoptin-associating proteins resulted in the detection of two types of proteins,

namely a Hou/Nmi-like protein and an IFP35-like protein. The apoptin-associating amino-acid sequences are homologous with the known Hou/Nmi amino-acid sequence or homologous with the known IFP35 amino-acid sequence. Hou/Nmi also share a homologous region (see below).

The determined DNA sequences of the two independent Hou/Nmi cDNA clones are shown in Figures 1 and 2, respectively. The amino acid sequence, derived from the detected DNA sequences is given in Figure 3. Remarkably, the complete open-reading frame (ORF) of the Hou-like protein was proven to be characterized.

The found DNA sequences of the three independent IFP35-like cDNA clones are shown in Figures 4, 5 and 6, respectively. Figure 7 shows the combination of the 2 independent IFP35 amino-acid sequences. The common part of these clones will associate with apoptin.

Interestingly, the C-terminus of Nmi shows homology to IFP35 (a.a. 102-288, 46% similarity; (Bao and Zervos, 1996, Bange et al, 1994). Actually, these data show that it is expected that our genetic yeast screen has resulted in these two apoptin-associating proteins, for they share a common homologous region.

Construction of an expression vector for the identification of the association of Apoptin and Hou/Nmi-like proteins and/or IFP35 in mammalian cells.

To study the association of Apoptin and the Hou/Nmi-like proteins and/or IFP35 in a mammalian cellular background, we have generated pSM2NT vectors containing the Hou/Nmi and/or IFP35 cDNA inserts. Another important feature of this approach is that we can prove that the cloned cDNAs indeed encode (Apoptin-associating) protein products.

The DNA plasmid pSM2NT contains the adenovirus 5 major late promoter (MLP) and the SV40 ori enabling high levels of expression of foreign genes in transformed mammalian cells, such as Cos cells.

Furthermore, the pSM2NT vector contains a Myc-tag (amino acids: EQKLISEEDL) which is in frame with the foreign-gene product. This Myc-tag enables the recognition of the e.g. Apoptin-associating proteins by means of the Myc-tag-specific 9E10 antibody.

The pSM2NT constructs expressing Myc-tagged Hou/Nmi and IFP35 were constructed as follows. The pACT-Hou/Nmi clone no.6 and pACT-IFP-35 no.51 were digested with the restriction enzyme XhoI and the requested cDNA inserts were isolated. The expression vector pSM2NT was digested with XhoI and treated with calf intestine alkline phosphatase and ligated to the subsequent isolated cDNA inserts. By sequence analysis, the pSM2NT clones containing the Hou/Nmi and IFP35 in the correct orientation were identified.

The expression of the Myc-tagged Hou/Nmi and IFP35 proteins was analyzed by transfection of Cos cells with plasmid pSM2NT-Hou/Nmi or pSM2NT-IFP35. As negative control, Cos cells were mock-transfected. Two days after transfection, the cells were lysed and Western-blot analysis was carried out using the Myc-tag-specific antibody 9E10. The Cos cells transfected with pSM2NT-Hou/Nmi were proven to synthesize a specific Myc-tagged Hou/Nmi product with the expected size of approximately 40 kDa. The lysate of the cells transfected with the plasmid encoding Myc-tagged IFP35 protein were shown to contain the supposed product size of about 26 kDa reacting with the Myc-tag-specific antibodies.
As expected, the lysates of the mock-transfected Cos cells did not contain a protein product reacting with the Myc-tag-specific antibodies.

These results indicate that we have been able to isolate cDNAs that are indeed able to produce a protein product with the ablity to associate to the apoptosis-inducing protein Apoptin.

Co-immunoprecipitation of Myc-tagged Hou/Nmi and IFP35 with Apoptin in a transformed mammalian cell system.

Next, we have analyzed the association of Apoptin and Hou/Nmi and/or IFP35 by means of co-immunoprecipitations using the Myc-tag-specific antibody 9E10. The 9E10 antibodies were shown not to bind directly to Apoptin, which enables the use of 9E10 for carrying out co-immuno-precipitations with (myc-tagged) Apoptin-associating proteins and Apoptin. To that end, Cos cells were co-transfected with plasmid pCMV-VP3 encoding Apoptin and with plasmid pSM2NT-Hou/Nmi encoding the Myc-tagged Hou/Nmi protein or with pSM2NT-IFP35 encoding the Myc-tagged IFP35. As negative control, we have transfected cells with Apoptin and a plasmid pSM2NT-LacZ encoding the myc-tagged beta-galactosidase, which does not associate with Apoptin.

Two days after transfection, the cells were lysed in a buffer consisting of 50 mM Tris (7.5), 250 mM NaCl, 5 mM EDTA, 0.1 % Triton X100, 1 mg/ml Na4P2O7 and freshly added protease inhibitors such as PMSF, Trypsine-inhibitor, Leupeptine and Na3VO4. The specific proteins were immuno-precipitated as described by Noteborn et al. (1998) using the Myc-tag-specific antibodies 9E10, and analyzed by Western blotting.

Staining of the Western blot with 9E10 antibodies and 111.3 antibodies, which are specifically directed against Apoptin, showed that the "total" cell lysates contained Apoptin and the Myc-tagged Hou/Nmi, IFP35 or beta-galactosidase product. Immunoprecipitation of the Myc-tagged Hou/Nmi and IFP35 products was accompanied by the immunoprecipatation of Apoptin product of 16 kDa. In contrast, immunoprecipitation of myc-tagged beta-galactosidase did not result in co-precipitation of the Apoptin protein.

In total, three independent immunoprecipitation experiments were carried out, which all showed the associating ability of Apoptin to the Hou/Nmi and IFP35 proteins.

These results indicate that besides the yeast background, both Hou/Nmi and IFP35 are able to specifically associate with Apoptin in a mammalian transformed cellular system.

Hou/Nmi-like proteins

The remarkable feature of apoptin-induced apoptosis is its tumor-specific activity. The fact that apoptin binds to Hou/Nmi-like proteins unravels this tumor/transformation-specific activity of apoptin. Below, the terms Hou/Nmi-like, Nmi, or Hou will be interchangeably used.

In this respect, the pattern of Nmi expression is interesting, since it is expressed at low levels in normal tissues, in contrast to its high levels of expression in transformed cell lines. Among eight cancer lines tested, highest levels were observed in four leukemia cell lines (Bao and Zervos, 1996).

In leukemias, a high expression of C-myc correlates with a high level of Nmi (HL-60, K562 and MOLT-4). The Nmi gene is located on chromosome 22, which is also involved in the t (9;22) translocation leading to the Bcr-Abl fusion protein, as seen in some leukemias (Rabbits, 1991, Sawyers and Deny, 1994).

Using a yeast genetic screen, Nmi was identified as a protein that binds to N-myc and C-myc. Myc proteins are important in the regulation of cell proliferation and differentiation. Together with ras or raf, myc can transform primary cells in culture. Nmi/Hou-like proteins will up-regulate the activity of Myc proteins via binding to them.

Up-regulation of Myc proteins has been described for Burkitt lymphomas, neuroblastomas and small cell lung carcinomas. Myc proteins contain a basic region, a helix-loop-helix (HLH) and a leucine zipper (Zip), and form homo- or heterodimers that can bind to specific DNA sequences and regulate transcription. Myc also forms heterodimers with Max. Myc/Max heterodimers activate transcription, whereas Max

homodimers repress transcription, thus antagonizing Myc's function (Evan and Littlewood, 1993).

Nmi was found to interact with N-myc, c-myc, Max, Mxi1 and other transcription factors that have HLH and/or Zip motifs. Interaction with N-myc and C-myc was confirmed by co-precipitation experiments (Bao and Zervos, 1996).

Induction of apoptosis through interference with the function of Nmi/Hou-like proteins.

Our results indicate that apoptin can change the Nmi/Hou-like-mediated proliferation (transformation/tumor-formation) activity into a Nmi/Hou-like-mediated apoptotic activity. Remarkably, this Nmi/Hou-like-mediated apoptotic activity will be specific for transformed/tumor cells, due to the very high level of Nmi/Hou in transformed cells in combination with over-expression of (proto-)oncogenes, such as Myc.

By means of transient transfection assays, it was shown that over-expression of the determined Hou-like protein (see Fig. 3) and apoptin did result in induction of apoptosis in normal VH10-, VH25-fibroblasts. In contrast to normal fibroblasts which over-expressed only apoptin. This result indicates that Hou-like proteins are an important factor in (apoptin-induced) apoptosis.

The presented data imply that interference with the function of Nmi/Hou-like proteins resulting in apoptosis can be used as a specific anti-tumor therapy, or therapies of related diseases, such as auto-immune diseases.

Characteristics of the apoptin-associating protein IFP35

The other apoptin-associating protein is IFP35, which is an interferon(IFN)-induced leucine zipper protein of 282 a.a., and has an apparent molecular mass of 35 kD. It was isolated by differential screening from HeLa cells that had been treated with IFN-γ (Bange et al., 1994).

IFP35 mRNA could be induced by IFN-γ in different human cell types, including fibroblasts, macrophages, and epithelial cells. It has a leucine zipper motif at the N-terminus, but it lacks an adjacent basic domain required for DNA binding. It has been suggested that these types of proteins negatively regulate bZIP transcription factors by forming non-functional heterodimers. IFP35 was shown to form homodimers (Bange et al., 1994).

Induction of apoptosis by interference of IFP35 in combination with Hou/Nmi-like proteins.

IFP35 is found in the cell nucleus, after interferon treatment and is expressed in a wide variety of cell types including fibroblasts, macrophages and epithelial cells (Bange et al., 1994).

In general, virus infections trigger interferon production. It is likely that a CAV infection and/or expression of apoptin will result in interferon up-regulation, which might result in the translocation of IFP35 or IFP35-like proteins into the nucleus. IFP35 will transport apoptin also to the nucleus, due to its association.

It seems likely that if apoptin is transported into the nucleus by IFP35 it will be able to associate with the IFP35-homologous region within Hou/Nmi-like proteins. This association will cause an aberrant regulation of Hou/Nmi-regulated genes, such as the oncogene Myc. Subsequently, the cells over-expressing Nmi/Hou-like proteins and oncogenes, such as Myc will undergo apoptosis.

Experimental evidence for IFP35 as an essential factor in (apoptin) apoptosis induction was derived from the following experiments. Normal VH10 cells over-expressing Hou/Nmi, IFP35 and apoptin underwent faster apoptosis than normal VH10 cells expressing Hou/Nmi and apoptin.

Conclusions

In conclusion, we have provided evidence that interference of specific factors with the function of Nmi/Hou, of IFP35 or of both will result in induction of apoptosis.

Therapies based on induction of apoptosis are possible if they succeed in the interference with the function of Nmi/Hou-like and/or of IFP35-like proteins. An example of such an interfering factor is apoptin. Another CAV-derived protein, which is known to induce apoptosis and also known to enhance apoptin activity is VP2 (Noteborn et al., 1997).

Other apoptin-associating proteins

The genetic yeast screen with pGBT-VP3 as bait plasmid and pACT plasmid containing cDNAs from transformed human B cells also delivered the protein filamin. The protein filamin is localized within lamellipodia and filopodia. Filamin is one of the cross-linking proteins of actin. It may play an additional role of linking the cytoskeleton to cell-substratum adhesion sites (Matsudaira, 1994).

Two independent filamin-like clones were found. The found associating amino acid sequence of the two filamin clones are shown in Figure 8.

To analyze into further detail the associating properties of Apoptin and filamin, we have co-expressed Myc-tagged filamin-like proteins by means of the pSM2NT vector (as described for Hou/Nmi and IFP35) in Cos cells together with Apoptin. Immunoprecipitation data clearly showed that 9E10 precipitates both filamin and Apoptin indicating that Apoptin associates to filamin in Cos cells.

Our data indicate that Apoptin associates with filamin in both yeast and transformed mammalian cells.

Production of polyclonal antibodies directed against Hou/Nmi- and IFP35-like proteins.

For the production of polyclonal antibodies against Hou/Nmi- and IFP35-like proteins putative immunogenic peptides were synthesized (Hou/Nmi peptide consists of the amino acids N/terminus-RNGGGEVDRVDYDRQ-C/terminus, and the IFP35 peptide of the aminoacids N/terminus-CQLRKELGDSPKDKVP-C/terminus; EuroGentec SA, Belgium). Subsequently, rabbits were injected with the specific peptides according the standard procedures of the manufacturer.

The serum derived from the rabbits injected with the Hou/Nmi peptide was shown to be specific for the above described Hou/Nmi products by means of immunofluoresence and Western-blot assays.

Serum from rabbits injected with the IFP35-specific peptide was proven to recognize specifically IFP35 encoded by the above described plasmid pSM2NT-IFP35.

These results imply that we have generated specific antibodies, which can be used for detecting the Apoptin-associating proteins Hou/Nmi and IFP35.

Description of the figures

Figure 1 shows the DNA sequence of the analysed region of the apoptin-associating clone Nmi/Hou-like No-1.

Figure 2 shows the DNA sequence of the analysed region of the apoptin-associating clone Nmi/Hou-like No-2.

Figure 3 shows the combination of the amino acids of the sequenced Nmi/Hou-like, derived from clones No-1 and No-2. In addition, the three C-terminal amino acids H-E-G of the multiple cloning site of pACT are given to illustrate that the Nmi/Hou-like amino acid sequence is in frame with the GAL4-activation domain. This feature proves that the Nmi/Hou-like region is indeed synthesized in yeast cells.

Figure 4 shows the DNA sequence of the analysed region of the apoptin-associating clone IFP35-like No-1.

Figure 5 shows the DNA sequence of the analysed region of the apoptin-associating clone IFP35-like No-2.

Figure 6 shows the amino acids of the sequenced region of the apoptin-associating clone IFP35-like No-3.

Figure 7 shows the combination of the amino acids of the sequenced IFP35-like clones No-2 and No-3. The fact that they overlap with each other implies that the common region of these three inserts associates with apoptin. In addition, part of the amino acid sequence of the known IFP35 is shown.

Figure 8 shows the amino acids of the sequenced region of the apoptin-associating clone Filamin No-1 and No-2. In addition, the three C-terminal amino acids H-E-G of the multiple cloning site of pACT are given to illustrate that the filamin-like amino acid sequence is in frame with the GAL4-

activation domain. This feature proves that the filamin-like region is indeed synthesized.

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CLAIMS

1. A recombinant and/or isolated nucleic acid molecule encoding at least a functional part of a member of the family of Nmi-like proteins or at least a functional part of a member of the family of Hou-like proteins or at least a functional part of a member of the family of IFP35-like proteins for use in the induction of apoptosis in a population of cells related to a pathological condition.

2. A use according to claim 1 wherein said nucleic acid molecule comprises at least a functional and specific part of the sequence of figure 1, 2, 4 or 5 or encoding an amino sequence of figure 6 or a sequence at least 60, preferably 70, preferably 90 % homologous with said functional and specific sequence or comprising a sequence hybridizing to any of the aforegoing sequences under stringent conditions.

3. Use according to claim 1 or 2 wherein said nucleic acid comprises an expression vector.

4. Use according to anyone of the aforegoing claims whereby said cells are provided with apoptosis inducing activity.

5. Use according to claim 4 whereby said apoptosis inducing activity is apoptin-like activity.

6. Use according to any of claims 1-5 wherein said nucleic acid is part of a gene delivery vehicle.

7. A recombinant and/or isolated nucleic acid molecule encoding an Nmi/Hou-like protein comprising at least a functional and/or specific part of the sequence of figure 1 or figure 2 or a sequence at least 60, preferably 70, more preferably 80% homologous therewith.

8. A recombinant and/or isolated nucleic acid molecule encoding an IFP35-like protein comprising at least a functional and/or specific part of the sequence of figure 4 or figure 5 or encoding at least a functional and/or specific part of the amino acid sequence of figure 6 or a sequence at least 60, preferably 70, more preferably 80% homologous therewith.

9. An expression vector comprising a recombinant nucleic acid molecule according to claim 7 and/or 8.

10. An expression vector according to claim 9 further comprising a sequence encoding apoptotic activity.

11. An expression vector according to claim 10 wherein said sequence encoding apoptotic activity encodes apoptin or a functional fragment and/or equivalent thereof.

12. A gene delivery vehicle comprising a recombinant nucleic acid molecule according to claim 7 or 8 or an expression vector according to anyone of claims 9-11.

13. A recombinant or isolated proteinaceous substance comprising at least a functional part of a member of the family of Nmi/Hou-like proteins or at least a functional part of a member of the family of Hou-like proteins for use in the induction of apoptosis in a population of cells related to a pathological condition.

14. An Nmi/Hou-like proteinaceous substance having at least a functional and/or specific part of the sequence of figure 3 or being encoded by a functional and/or specific part of the sequence of figure 1 or figure 2 or being at least 60, preferably 70, preferably 80% homologous to at least a functional and/or specific part of the sequence of figure 3 or being at least 60, preferably 70, preferably 80% homologous to a protein encoded by at least a functional and/or specific part of the sequence of figure 1 or figure 2.

15. A recombinant or isolated proteinaceous substance comprising at least a functional part of a member of the family of Nmi/Hou-like proteins or at least a functional part of a member of the family of Hou-like proteins for use in the induction of apoptosis in a population of cells related to a pathological condition.

16. An IFP35-like proteinaceous substance having at least a functional and/or specific part of the sequence of figure 6 or 7 or being encoded by a functional and/or specific part of the sequence of figure 4 or figure 5 or being at least 60, preferably 70, preferably 80% homologous to at least a

functional and/or specific part of the sequence of figure 6 or 7 or being at least 60, preferably 70, preferably 80% homologous to a protein encoded by at least a functional and/or specific part of the sequence of figure 4 or figure 5.

17. A method for inducing apoptosis in cells comprising providing said cells with Nmi/Hou-like protein activity and/or IFP-35-like activity together with apoptin-like activity.

18. Use of apoptin to find proteinaceous substances associated with apoptosis.

GGGGGATCATGGAAGCTGATAAAGATGACACACAACAAATTCTTAAGGAGCATTCGCCAGATGAA
TTTATAAAAGATGAACAAAATAAGGGACTAATTGATGAAATTACAAAGAAAAATATTCAACTAAA
GAAGGAGATCCAAAAGCTTGAAACGGAGTTACAAGAGGCTACCAAAGAATTCCAGATTAAAGAGG
ATATTCCTGAAACAAAGATGAAATTCTTATCAGTTGAAACTCCTGAGAATGACAGCCAGTTGTCA
AATATCTCCTGTTCGTTTCAAGTGAGCTCGAAAGTTCCTTATGAGATACAAAAAGGACAAGCACT
TATCACCTTTGAAAAAGAAGAAGTTGCTCAAAATGTGGTAAGCATGAGTAAACATCATGTACAGA
TAAAAGATGTAAATCTGGAGGTTACGGCCAAGCCAGTTCCATTAAATTCAGGAGTCAGATTCCAG
GTTTATGTAGAAGTTTCTAAAATGAAAATCAATGTTACTGAAATTCCTGACACATTGCGTGAAGA
TCAAATGAGAGACAAACTAGAGCTGAGCTTTTCAAAGTCCCGAAATGGGAGGCGGAGANGTGGAC
CGCGTGGGACTATGACAGACAGTCCGGGAGTGCAGTCATCACGTTTGGNGGAGATTGGGAGTGGC
TGACANNN

Figure 1

Hou c6/#1

CGGAGTTACAAGAGGCTACCAAAGAATTCCAGATTAAAGAGGATATTCCTGAAACAAAGATGAAA
TTCTTATCAGTTGAAACTCCTGANAATGACAGCCAGTTGTCAAATATCTCCTGTTCGTTTCAAGG
TGAGCTCGAAAGTTCCTTATGAGATACAAAAAGGACAATGCACTTATCACCTTTGAAAAAGGAAG
AAGTTGCTCAAAATGTGNGTAANGCATGAGTAAACATCATGTACAGATAATAAGATGTAAATCTG
GAGGTTACGGCCAAAGCCAAGTTCCATTAATATTCAAGGAGTCANGATTCCAGNGTTTATGCTAG
AANGTTTCTAAAAATGANAATCAATGGTTACTGGAAATTCCTGGACACATTGCGNTGAAAGATCA
AGATGACGAAGACAAACTAAGAAGCTGAGCTTTTCAAAAGTCCCGAAANATGGAAGAGCGGTAGA
GGGTGGNACCGCGTGNGANCTATGACAAGACAAGNCCGGGGAAGNTGCAGTCCATCACGTTTGTN
NGAAGATTGGANGTNGGCTGACCAANGAATTTTGAAAAAGGAGANGAATTACCCCTCTTTANGAG
TAANATCAAAACCCTGCCATAANAAGTTNACTGGTTTCNCCCATTACACAGNAN
TTACANNTTGANCAANANTANNCAGGATAATTTNCAGGGGAANAATCTNAAGNATGGCAAGNTGA
CTTCTGGACAANGGT

Figure 2

Hou c17/#2

```
c6/#1       1 HEGRGIMEADKDDTQQILKEHSPDEFIKDEQNKGLIDEITKKNIQLKKEIQKLETELQEA
Hou/Nmi     1 -------MEADKDDTQQILKEHSPDEFIKDEQNKGLIDEITKKNIQLKKEIQKLETELQEA

c6/#1      61 TKEFQIKEDIPETKMKFLSVETPENDSQLSNISCSFQVSSKVPYEIQKGQALITFEKEEV
Hou/Nmi    55 TKEFQIKEDIPETKMKFLSVETPENDSQLSNISCSFQVSSKVPYEIQKGQALITFEKEEV

c6/#1      121 AQNVVSMSKHHVQIKDVNLEVTAKPVPLNSGVRFQVYVEVSKMKINVTEIPDTLREDQMR
Hou/Nmi    115 AQNVVSMSKHHVQIKDVNLEVTAKPVPLNSGVRFQVYVEVSKMKINVTEIPDTLREDQMR

c6/#1       181 DKLELSFSKSRNGRRRCGPRGTMTDSPGVQSSRLVEIGS----------------------
Hou/Nmi     175 DKLELSFSKFRNGGGEV.DRVDYDRQSGSAVITFVEIGV-DKILKKKEYPLYINQTCHRV

c6/#1       221 ------------------------------------------------------------
Hou/Nmi     234 TVSPYTEIHLKKYQIFSGTSKRTVLLTGMEGIQMDEEIVEDLINIHFQRAKNGGGEVDVV

c6/#1       221 ---------------
Hou/Nmi     294 KCSLGQPHIAYFEE
```

Figure 3

AGCAGGTGCTGCAACAAAAGGAGCACACGATCAACATGGAGGAGTGCCGGCTGCGGGTGCAGGTC
CAGCCCTTGGAGCTGCCCATGGTCACCACCATCCAGGTGTCCAGCCAGTTGAGTGGCCGGAGGGT
GTTGGTCACTGGATTTCCTGCCAGCCTCAGGCTGAGTGAGGAGGAGCTGCTGGACAANCTANAGA
TCTTCTTTGGCAAGACTAGGAACGGAGGTGGCNATGTGGACNTTCGGGANCTACTGCCAGGGANT
GTCATGCTGGGGTTTGCTAGGGATGGAGTGGCTCANCGTCTGTGCCAAATCGGCCATTTCACAGT
GCCACTGGGTGGGCAGCANGTCCCTCTGAGAGTCTCTCCGTATGTGAATGGGGANATCCAGANGG
CTGANATCAGGTCNCAGCCANTTCCCCGCTCGGTACTGGTGCTCAACATTCCTGATATCTTGGAT
GGCCCGGAGCTGCATGACGTCCTGGANATCCACTTCCAGAANCCCACCCGCGGGGGCGGAGATGT
AAGACGCCCTGACAGTCGTACCCCAAGGACAACAGGGCCTAACAGTCTTCACCTCCTGAATCAAG
GCTANGGGCCTCCCCCTTCTCATCCTCCCCACCCCCCCCGCCAAAGGTTCTCAANACTGGGCCTG
GGCTTTNTG

Figure 4

IFP35 c14/#1

CCAAAGTGGCTGAGCAGGTGCTGCAACAAAAGGAGCACACGATCAACATGGAGGAGTGCCGGCTGCGGGTGC
AGGTCCAGCCCTTGGAGCTGCCCATGGTCACCACCATCCAGGTGTCCAGCCAGTTGAGTGGCCGGAGGGTGT
TGGTCACTGGATTTCCTGCCAGCCTCAGGCTGAGTGAGGAGGAGCTGCTGGACAAGCTAGAGATCTTCTTTG
GCAAGACTAGGAACGGAGGTGGCGATGTGGACGTTCGGGAGCTACTGCCAGGGAGTGTCATGCTGGGGTTTG
CTAGGGATGGAGTGGCTCAGCGTCTGTGCCAAATCGGCCAAGTTCACAGTGCCACTGGGTGGGCANCAAGTC
CCTCTGAGAGTCTCTCCGTATGTGAATGGGGAGATCCAGAAGGCTGAGATCAGGTCGCANCCAGTTCCCCNC
TCGGTACTGGGTGCTCAACATTCCTGATATCTTGGATTGGCCCGGAGCTGCATNACGTCCTGGANATCAACT
TCANAAGCCCACCCGCCGGGGCNGNGAGGTANAAGGCCTGACATCNTTACCCCAAAGGACAGCATGGNCCTA
ACAGTCCTCACCTCCNAATCANGCTNNGGGGCTNCCCTTCTANCNTCCCCAACTG

Figure 5

IFP35 c33/#2

SUBSTITUTE SHEET (RULE 26)

GGATCCACTGCCCTCTGCTTGCGGGCTCTGCTCTGATCACCTTTGATGACCCCAAAGTGGCTGAG
CAGGTGCTGCAACAAAAGGAGCACACGATCAACATGGAGGAGTGCCGGCTGCGGGTGCAGGTCCA
GCCCTTGGAGCTGCCCATGGTCACCACCATCCAGGTGATGGTGTCCAGCCANTTGAGTGGCCGGA
GGGTGTTGGTCACTGGATTTCCTGCCAGCCTCAGGCTGANTGAGGAGGAGCTGCTGGACAAGCTA
TGAGATCTTCTTTGGCAANACTANGAACGGANGTGGCGATGTGGACGTTCGGGAGCTACTGCCAG
GGAGTGTCATGCTGGGGTTTGCTACGGATGGAGTGGCTCAGCGTCTGTGCCAAATCGGCCAGTTC
ACAAGTGCCACTGGGTGGGCAGCAAGTCCCTCTGAGAGTCTCTCCGTATGTGANTGGNGAGATCA
GAATGCTGANATTAAGTCGCATCCAATTCCTCGCTCNGGTACTGGTGCTCANNATCCTGANATCT
TGGATTGGCCCCNGANTNCATGANATCTGGNAGATTCAATTNCANAAGTCCANCCNNCNGNGNCG
GGAAGTANANGCCCGANANTTCNTNNCNTANGGNCAGCANNGCCTG

Figure 6

IFP35 c51/#3

SUBSTITUTE SHEET (RULE 26)

```
33ifn          1 ------------------------------------------------------------
In35_Human     1 MSAPLDAALHALQEEQARLKMRLWDLQQLRKELGDSPKDKVPFSVPKIPLVFRGHTQQDP
C51            1 ------------------------------------------------------------

33ifn          1 --------------------------HEGPKVAEQVLQQKEHTINMEECRLRVQVQPLELPM
In35_Human    61 EVPKSLVSNLRIHCPLLAGSALITFDDPKVAEQVLQQKEHTINMEECRLRVQVQPLELPM
C51            1 --------HEGRIHCPLLAGSALITFDDPKVAEQVLQQKEHTINMEECRLRVQVQPLELPM

33ifn         37 VTTIQ..VSSQLSGRRVLVTGFPASLRLSEEELLDKLEIFFGKTRNGGGDVDVRELLPGS
In35_Human   121 VTTIQ..VSSQLSGRRVLVTGFPASLRLSEEELLDKLEIFFGKTRNGGGDVDVRELLPGS
C51           54 VTTIQVMVSSXLSGRRVLVTGFPASLRLXEEELLDKL*DLLWQXXERXWRC.........

33ifn         95 VMLGFARDGVAQRLCQIGQVHSATGWASSPSESLSVQEWGDPEG----------------
In35_Human   179 VMLGFARDGVAQRLCQIGQFTVPLGGQQVPLRVSPYVNGEIQKAEIRSQPVPRSVLVLNI
C51          104 .....GRSGATARECHAGVCYGWSGSASVPNRPVHKGHWVGSKSL*ESLRM*KXRSEC*X

33ifn        139 ------------------------------------------------------------
In35_Human   239 PDILDGPELHDVLEIHFQKPTRGGGGRGPDSRTPRTAGPSSLHL-----------
C51          156 *VASNSSLXYWCSXS*XLGLAPXXMXSGRFNXXSPXXXXGKXXPXXSXXXXSXA
```

Figure 7

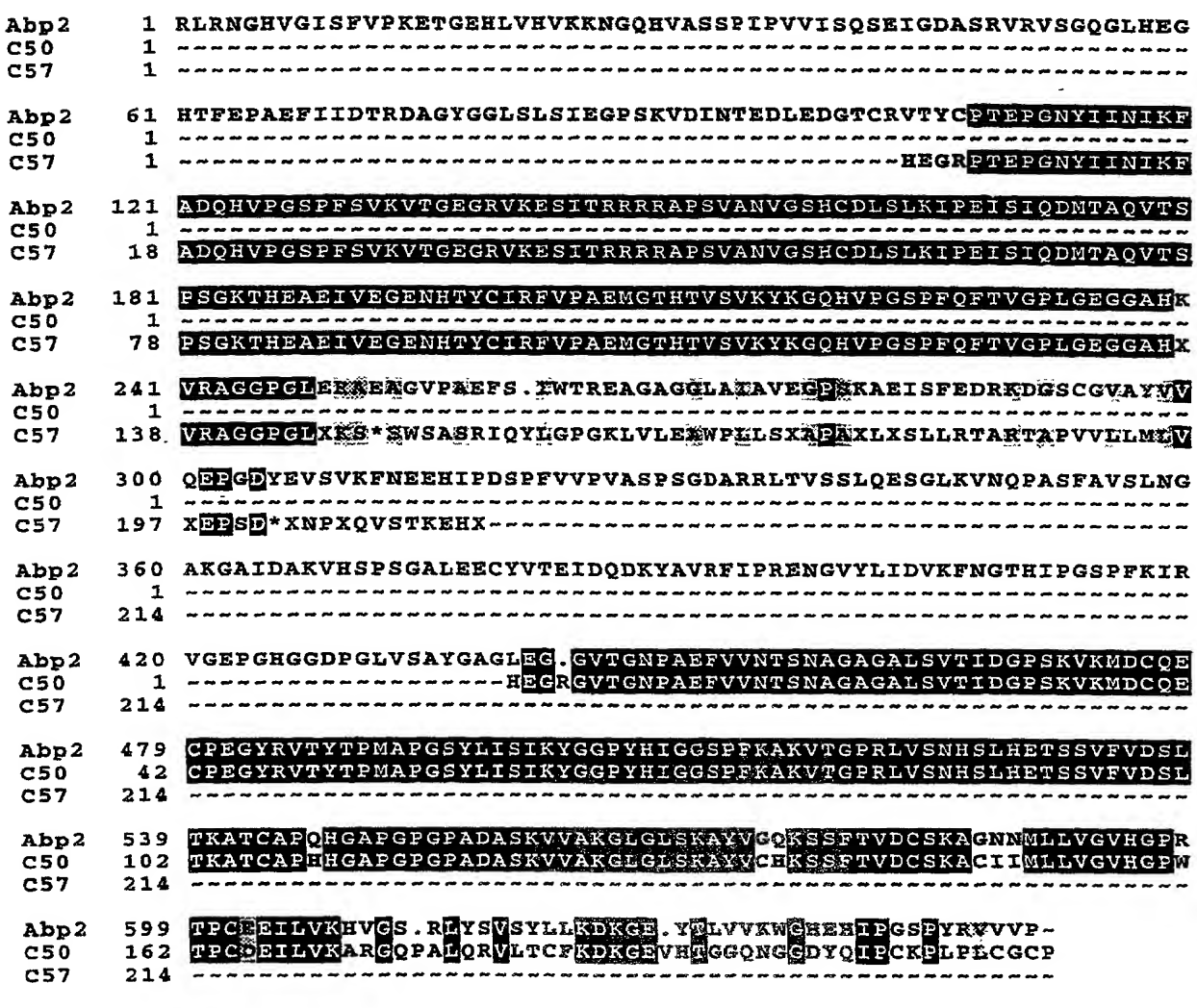
Abp2 1 RLRNGHVGISFVPKETGEHLVHVKKNGQHVASSPIPVVISQSEIGDASRVRVSGQGLHEG
C50 1
C57 1
Abp2 61 HTFEPAEFIIDTRDAGYGGLSLSIEGPSKVDINTEDLEDGTCRVTYCPTEPGNYIINIKF
C50 1
C57 1 HEGRPTEPGNYIINIKF
Abp2 121 ADQHVPGSPFSVKVTGEGRVKESITRRRRAPSVANVGSHCDLSLKIPEISIQDMTAQVTS
C50 1
C57 18 ADQHVPGSPFSVKVTGEGRVKESITRRRRAPSVANVGSHCDLSLKIPEISIQDMTAQVTS
Abp2 181 PSGKTHEAEIVEGENHTYCIRFVPAEMGTHTVSVKYKGQHVPGSPFQFTVGPLGEGGAHK
C50 1
C57 78 PSGKTHEAEIVEGENHTYCIRFVPAEMGTHTVSVKYKGQHVPGSPFQFTVGPLGEGGAHX
Abp2 241 VRAGGPGLEKKEAGVPAEFS.IWTREAGAGGLAIAVEGPSKAEISFEDRKDGSCGVAYVV
C50 1
C57 138 VRAGGPGLXKS*SWSASRIQYLGPGKLVLEKWPLLSXAPAXLXSLLRTARTAPVVLLMLV
Abp2 300 QEPGDYEVSVKFNEEHIPDSPFVVPVASPSGDARRLTVSSLQESGLKVNQPASFAVSLNG
C50 1
C57 197 XEPSD*XNPXQVSTKEHX
Abp2 360 AKGAIDAKVHSPSGALEECYVTEIDQDKYAVRFIPRENGVYLIDVKFNGTHIPGSPFKIR
C50 1
C57 214
Abp2 420 VGEPGHGGDPGLVSAYGAGLEG.GVTGNPAEFVVNTSNAGAGALSVTIDGPSKVKMDCQE
C50 1 HEGRGVTGNPAEFVVNTSNAGAGALSVTIDGPSKVKMDCQE
C57 214
Abp2 479 CPEGYRVTYTPMAPGSYLISIKYGGPYHIGGSPFKAKVTGPRLVSNHSLHETSSVFVDSL
C50 42 CPEGYRVTYTPMAPGSYLISIKYGGPYHIGGSPFKAKVTGPRLVSNHSLHETSSVFVDSL
C57 214
Abp2 539 TKATCAPQHGAPGPGPADASKVVAKGLGLSKAYVGQKSSFTVDCSKAGNNMLLVGVHGPR
C50 102 TKATCAPHHGAPGPGPADASKVVAKGLGLSKAYVCHKSSFTVDCSKACIIMLLVGVHGPW
C57 214
Abp2 599 TPCEEILVKHVGS.RLYSVSYLLKDKGE.YTLVVKWGHEHIPGSPYRVVVP-
C50 162 TPCDEILVKARGQPALQRVLTCFKDKGEVHTGGQNGGDYQIPCKPLPLCGCP
C57 214

Figure 8

PATENT

ATTORNEY DOCKET NO. LEBV.006.01US

COMBINED INVENTOR
DECLARATION AND POWER OF ATTORNEY

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name, I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Molecules Interacting with Apoptin

the specification of which

[X] Is attached hereto.
[X] Was filed on Monday, June 5, 2000 as Attorney Docket No. LEBV.006.01US

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information known to me to be material to patentability of this application as defined in Title 37, Code of Federal Regulations, §1.56 and, if applicable, all such information under 37 CFR § 1.56 which became available between the national or PCT International filing date of the prior application and the filing date of this application.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)			Priority Claimed	
97203781.6	EP	December 3, 1997	[X]	[ ]
(number)	(Country)	(Day/Month/Year Filed)	Yes	No

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 USC §112 I acknowledge the duty to disclose all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56, which became available between the filing date of the prior application and the national or PCT international filing date of this application:

PCT/NL98/00687*	December 3, 1998	Published
(Application Serial No.)	(Filing Date)	(Status)

* designates the U.S.

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as my attorneys or agents with full power of substitution and revocation to prosecute my above-identified application for Letters Patent and to transact all business in the Patent Office connected therewith.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18, United States Code, §1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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